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Nicotine and Ethanol Cooperate to Enhance Ventral Tegmental Area AMPA Receptor Function via $\alpha 6$ -Containing Nicotinic Receptors

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Abstract

Nicotine+ethanol co-exposure results in additive and/or synergistic effects in the ventral tegmental area (VTA) to nucleus accumbens (NAc) dopamine (DA) pathway, but the mechanisms supporting this are unclear. We tested the hypothesis that nAChRs containing $\alpha 6$ subunits ($\alpha 6^*$ nAChRs) are involved in the response to nicotine+ethanol co-exposure. Exposing VTA slices from C57BL/6 WT animals to drinking-relevant concentrations of ethanol causes a marked enhancement of α -amino-3-hydroxy-5-methyl-isoxazolepropionic acid (AMPA) receptor (AMPA) function in VTA neurons. This effect was sensitive to α -conotoxin MII (an $\alpha 6\beta 2^*$ nAChR antagonist), suggesting that $\alpha 6^*$ nAChR function is required. In mice expressing hypersensitive $\alpha 6^*$ nAChRs ($\alpha 6L9S$ mice), we found that lower concentrations (relative to C57BL/6 WT) of ethanol were sufficient to enhance AMPAR function in VTA neurons. Exposure of live C57BL/6 WT mice to ethanol also produced AMPAR functional enhancement in VTA neurons, and studies in $\alpha 6L9S$ mice strongly suggest a role for $\alpha 6^*$ nAChRs in this response. We then asked whether nicotine and ethanol cooperate to enhance VTA AMPAR function. We identified low concentrations of nicotine and ethanol that were capable of strongly enhancing VTA AMPAR function when co-applied to slices, but that did not enhance AMPAR function when applied alone. This effect was sensitive to both varenicline (an $\alpha 4\beta 2^*$ and $\alpha 6\beta 2^*$ nAChR partial agonist) and α -conotoxin MII. Finally, nicotine+ethanol co-exposure also enhanced AMPAR function in VTA neurons from $\alpha 6L9S$ mice. Together, these data identify $\alpha 6^*$ nAChRs as important players in the response to nicotine+ethanol co-exposure in VTA neurons.

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Keywords

nicotine; alcohol; addiction; dopamine; glutamate; co-exposure

1. Introduction

Co-abuse of nicotine and alcohol is exceedingly common (WHO, 2011); many alcoholics are also smokers, and a majority of smokers drink large quantities of alcohol (Bobo, 1992; DiFranza and Guerrera, 1990; Grant et al., 2004; Miller and Gold, 1998). The prevalence of tobacco+alcohol co-addiction suggests the existence of an interaction between nicotine and alcohol in the brain. Drinking alcohol increases smoking behavior, craving to smoke, and the subjective rewarding effects of smoking (Harrison et al., 2009; King and Epstein, 2005; Mitchell et al., 1995; Rose et al., 2004). The converse is also true; exposure to nicotine increases alcohol consumption (Doyon et al., 2013). Although there is clearly robust evidence for interactions between nicotine and alcohol, molecular and cellular mechanisms supporting this interaction are not well understood.

Transitioning from the drug-naïve state to the addicted state involves alterations in neuronal function, and altered glutamate receptor activity plays an outsized role in this (Kauer and Malenka, 2007). VTA neurons express multiple types of glutamate-activated cation channels, with AMPARs and N-methyl D-aspartate (NMDA) receptors (NMDARs) being the most important of these. Drug-induced behavioral changes, such as the acquisition of self-administration, are driven by synaptic changes (Luscher and Malenka, 2011) that support long-term potentiation (LTP) of excitatory transmission at VTA neurons (Argilli et al., 2008; Borgland et al., 2004; Dong et al., 2004). This LTP is caused, at least in part, by increased expression and/or enhanced function of surface AMPARs (Argilli et al., 2008; Borgland et al., 2004; Dong et al., 2004; Luscher and Malenka, 2011; Saal et al., 2003; Ungless et al., 2001). Enhanced AMPAR function in VTA neurons occurs 1) when VTA slices from naïve animals are exposed to nicotine (Jin et al., 2011; Mao et al., 2011), and 2) following intraperitoneal (i.p.) injections of nicotine into mice or rats (Gao et al., 2010; Saal et al., 2003). Ethanol exposure also enhances VTA neuron AMPAR function (Stuber et al., 2008), suggesting that AMPAR function may be a site where nicotine and ethanol converge to modulate reward system function during nicotine+ethanol co-exposure.

The VTA is comprised of groups of neurons producing DA, gamma-aminobutyric acid (GABA), and/or glutamate (Creed et al., 2014; Hnasko and Edwards, 2012; Hnasko et al., 2012; Sulzer et al., 1998). VTA neurons express high levels of nicotinic acetylcholine receptors (nAChRs), which are pentameric ligand-gated cation channels. These receptors are the molecular target of nicotine from tobacco products. nAChRs are either homomeric (containing five $\alpha 7$ subunits) or heteromeric (containing two α subunits, two β subunits, and a fifth accessory subunit that is either an α or a β subunit). VTA neurons express two main, high-sensitivity (Calabresi et al., 1989; Salminen et al., 2007; Salminen et al., 2004) nAChR subtypes: nAChRs containing the $\alpha 4$ subunit and nAChRs containing the $\alpha 6$ subunit. $\alpha 4^*$ (* denotes the potential presence of other subunits besides the indicated subunit) nAChRs are expressed widely throughout the brain, including expression in both DAergic and GABAergic VTA neurons (Champtiaux et al., 2003; Marubio et al., 2003; Nashmi et al.,

2007; Ross et al., 2000). In contrast, $\alpha 6^*$ nAChRs are restricted to only a few brain areas (le Novère et al., 1999; Lena et al., 1999; Mackey et al., 2012; Whiteaker et al., 2000), one being the midbrain DA system. Unlike $\alpha 4^*$ nAChRs, $\alpha 6^*$ nAChRs are only expressed in DA neurons within the VTA and not in VTA GABA neurons (Drenan et al., 2008; Mackey et al., 2012). $\alpha 4^*$ (Tapper et al., 2004) and $\alpha 6^*$ (Pons et al., 2008) nAChRs are strongly implicated in the rewarding and reinforcing properties of nicotine, and emerging evidence points to a role for nAChRs containing both $\alpha 4$ and $\alpha 6$ subunits ($\alpha 4\alpha 6^*$) (Drenan et al., 2010; Engle et al., 2013; Liu et al., 2012).

$\alpha 4^*$ and $\alpha 6^*$ nAChRs also play an important role in the response to ethanol. $\alpha 4$ nAChR subunits are necessary for ethanol-induced reward-like behavior in mice (Liu et al., 2013). Infusion of an $\alpha 6$ nAChR antagonist into the VTA reduced both voluntary ethanol drinking (Larsson et al., 2004) and operant responding for ethanol (Lof et al., 2007), supporting a role for $\alpha 6^*$ nAChRs in ethanol reward. We recently corroborated this by showing that enhanced $\alpha 6^*$ nAChR activity increases ethanol consumption and reward-like behavior (Powers et al., 2013). In this study, we used electrophysiology, pharmacology, and genetically modified mice to further explore the role of $\alpha 6^*$ nAChRs in the response to ethanol. Finally, in the current study, we tested the hypothesis that $\alpha 6^*$ nAChRs are important for the response to nicotine+ethanol co-exposure.

2. Materials and Methods

2.1 Materials

Unless specified otherwise, all chemicals were purchased from Sigma-Aldrich. Tocris Biosciences was the source of tetrodotoxin. α -Conotoxin MII (α CtxMII) was synthesized as in previously published methods (Azam et al., 2010).

2.2 Mice and *in vivo* Drug Administration

All experiments with animals were conducted in accordance with the guidelines for the care and use of animals provided by the National Institutes of Health Office of Laboratory Animal Welfare. All protocols were approved by the Purdue University Institutional Animal Care and Use Committee. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to *in vivo* techniques when available. Mice were housed at 22°C and kept on a standard 12 hour light/dark cycle. Food and water was available *ad libitum*. Mice were weaned and group housed with same sex littermates on postnatal day 21. Tail biopsies were collected 21–28 days after birth and used to determine the genotype of the mice via PCR analysis as previously described (Drenan et al., 2008). Male and female $\alpha 6$ L9S mice and their non-transgenic (non-Tg) littermates were 8–16 weeks old at the time experiments were conducted. No differences were noted between results obtained from male versus female mice, so results were pooled.

$\alpha 6$ L9S mice were generated as previously described (Drenan et al., 2008). A Leu9' to Ser mutation was introduced into the $\alpha 6$ nAChR subunit gene, *Chrna6*, located in a mouse bacterial artificial chromosome (BAC). This mutant BAC DNA was injected into FVB/N embryos and embryos were implanted into pseudopregnant Swiss-Webster surrogates. The insertion site within the mouse genome is unknown. Because founder animals were back-

crossed to C57BL/6 mice for more than 12 generations it is expected that over 90% of the $\alpha 6$ L9S mouse genome is C57BL/6. However, FVB/N allelic DNA near the insertion site is probable. The L9S mutation renders $\alpha 6^*$ nAChRs hypersensitive to ligands such as nicotine and acetylcholine. $\alpha 6^*$ nAChRs in these mice are 10- to 100-fold more sensitive than non- $\alpha 6^*$ nAChRs, depending on the assay being used (Cohen et al., 2012; Drenan et al., 2010; Drenan et al., 2008). Previous studies confirm that $\alpha 6^*$ nAChRs in $\alpha 6$ L9S mice are expressed in the expected brain regions (and not in unexpected regions) and at the expected levels (Drenan et al., 2010; Drenan et al., 2008).

Prior to receiving intraperitoneal (i.p.) injections, mice were gently handled once a day for three days to habituate them to handling. The next day, mice were scruffed and given a mock injection. One day prior to the experiment, mice were scruffed and given a saline injection (i.p.). On the experimental day, mice were given an i.p. ethanol (or vehicle) injection. Ethanol was diluted in 0.9% saline and administered at a dose of either 0.5 or 2.0 g/kg. One hour after the injection mice were killed for brain slice preparation.

2.3 Brain Slice Preparation

Brain slices were prepared as previously described (Engle et al., 2012). Briefly, mice were anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and transcardially perfused with oxygenated (95% O₂ / 5% CO₂), 4°C *N*-methyl-D-glucamine (NMDG)-based recovery solution (in mM: 93 NMDG, 2.5 KCl, 1.2 NaH₂PO₄, 30 NaHCO₃, 20 HEPES, 25 glucose, 5 Na⁺ ascorbate, 2 thiourea, 3 Na⁺ pyruvate, 10 MgSO₄•7H₂O, and 0.5 CaCl₂•2H₂O; 300–310 mOsm; pH 7.3–7.4). Brains were dissected and immediately placed in oxygenated 4°C NMDG recovery solution for 1 min before cutting 200 μ m coronal brain slices with a vibrating microslicer. Slices were transferred to oxygenated 33°C NMDG recovery solution for 12 min before being held until recording at room temperature in HEPES holding solution (in mM: 92 NaCl, 2.5 KCl, 1.2 NaH₂PO₄, 30 NaHCO₃, 20 HEPES, 25 glucose, 5 Na⁺ ascorbate, 2 thiourea, 3 Na⁺ pyruvate, 2 MgSO₄•7H₂O, and 2 CaCl₂•2H₂O; 300–310 mOsm; pH 7.3–7.4).

2.4 Patch Clamp Electrophysiology

Patch clamp electrophysiology was carried out as previously described (Engle et al., 2012). A brain slice containing the VTA was placed in a recording chamber. Oxygenated artificial cerebrospinal fluid recording solution (in mM: 124 NaCl, 2.5 KCl, 1.2 NaH₂PO₄, 24 NaHCO₃, 12.5 glucose, 2 MgSO₄•7H₂O, and 2 CaCl₂•2H₂O; 300–310 mOsm; pH 7.3–7.4) containing tetrodotoxin (0.5 μ M) and picrotoxin (75 μ M) was heated to 32°C and continuously superfused over the slice at 1.5–2.0 ml/min. VTA DA neurons were found at coordinates of approximately –3.5 mm from bregma, 4.0–4.5 mm ventral to the surface, and 0.5–1.0 mm from the midline. Up to 96% of neurons in this area are expected to be DA neurons (tyrosine hydroxylase immunoreactive) (Lammel et al., 2008). Cells were visualized with a Nikon FN-1 upright microscope using infrared or visible differential interference contrast (DIC) optics. A programmable microelectrode puller was used to make patch electrodes from borosilicate glass capillary tubes. The tip resistance of patch electrodes used was between 4.5–8.0 M Ω when filled with internal pipette solution (in mM: 117 CsCH₃SO₃, 20 HEPES, 0.4 EGTA, 2.8 NaCl, 5 TEA-Cl, 2.5 MgATP, 0.1 spermine, and 0.25 MgGTP;

pH adjusted to 7.25 with Tris base; osmolarity adjusted to 290 mOsm with sucrose). An Axopatch 200B amplifier, a 16-bit Digidata 1440A A/D converter, and pCLAMP 10.3 software were used to acquire whole-cell recordings. Data were sampled at 5 kHz and low-pass filtered at 1 kHz. The junction potential between the patch pipette and the bath solution was nulled immediately prior to the formation of a gigaseal. Series resistance was uncompensated.

To examine AMPAR function, AMPA was locally applied to cells using a Picospritzer III as previously described (Engle et al., 2012; Engle et al., 2013). Briefly, a micropipette was filled with AMPA and mounted onto a single-dimension piezoelectric translator attached to a micromanipulator. AMPA was applied to the cells using a 250-millisecond pressure (12 psi) ejection triggered by protocols run in pClamp10. The protocol was programmed to move the AMPA-filled pipette to a pre-determined location (approximately 20–40 μm from the cell being recorded), trigger the pressure ejection, and retract the pipette to a distance 100 μm from the cell. This setup is preferred over manual control of the drug pipette because it results in enhanced reproducibility and reduced receptor desensitization (Engle et al., 2012). The drug-filled pipette was initially placed ~ 40 μm from the recorded cell and cell movement was monitored during drug exposure. If there was not sufficient cell movement, indicating the cell was not fully exposed to the drug, the drug pipette was moved closer to the cell and the recording repeated. The rise time of the AMPA-evoked current is another indication of an acceptable drug application. Slow rise times correlate with insufficient cell movement and indicate the AMPA-filled pipette was not close enough to the recorded cell. Data are also unacceptable when a stable seal was not maintained because the AMPA-filled pipette was too close to the recorded cell.

AMPA to NMDA ratio recordings were carried out as previously described (Etherton et al., 2011; Mao et al., 2011), but with minor modifications. Coronal slices were used (as for AMPA-evoked currents), neurons were chosen as described above, and whole-cell recordings were established using the following internal solution: (in mM) 117 CsCH_3SO_3 , 20 HEPES, 0.4 EGTA, 2.8 NaCl, 5 TEA-Cl, 2.5 MgATP, and 0.25 MgGTP; pH adjusted to 7.25 with Tris base; osmolarity adjusted to 290 mOsm with sucrose. A concentric bipolar stimulating electrode (FHC cat. #CBAPC75) was placed 100–200 μm lateral to the recorded cell, typically in/around the medial lemniscus in the ipsilateral hemisphere. Synaptic responses were stimulated (250 to 750 μA , 40 μs , 0.1 Hz) such that evoked EPSCs were $\sim 50\%$ of maximum amplitude. First, the cell was held at a command voltage of -70 mV, and an EPSC was evoked. Because there is little to no current through NMDA receptors at a membrane potential of -70 mV (due to Mg^{2+} block), the response at -70 mV – and therefore the time to peak – is likely to be completely mediated by AMPARs. Five sweeps were recorded at -70 mV, and the average time-to-peak was noted and subsequently used to measure AMPAR-mediated EPSCs at $+40$ mV. Next, cells were depolarized to a command voltage of $+40$ mV and membrane current was allowed to stabilize for several minutes prior to evoking EPSCs. EPSCs were again evoked, and 5 sweeps were averaged. The amplitude of the AMPA-mediated component at $+40$ mV was estimated to be the membrane current value at the time corresponding to when the evoked EPSCs peaked when the command voltage was -70 mV. The NMDA component was derived by averaging the membrane current value during a 10 msec window that began 40 msec after the time of the peak

AMPA response. Because AMPA components of evoked EPSCs decay to baseline well before 40 msec, this window accurately isolates the NMDA-mediated component of the evoked EPSC. AMPA components at +40 mV and NMDA components at +40 mV were used to derive an AMPA/NMDA ratio for each cell. This method was shown to yield results congruent with those obtained using 2-amino-5-phosphonopentanoic acid (AP-5) to pharmacologically isolate NMDA and AMPA components of the evoked EPSC in recordings of nicotine-evoked plasticity from VTA DA neurons. In a subset of cells, we verified our measured AMPA/NMDA ratios by using AP-5 to pharmacologically isolate NMDA and AMPA components (data not shown). For all recordings in VTA, one or two slices per animal were used, and data were obtained from between one and six cells per slice.

2.5 Statistical Analyses

Concentration-response data are presented at mean $\pm$ SEM. All other data plots are presented as box/whisker plots, with the middle line denoting the median, boxes enclosing the middle quartiles, and whiskers extending from minimum to maximum for each data set. For electrophysiology trace data, multiple traces were averaged and a mean current trace was generated and is shown (black lines). To convey variability in electrophysiology data, SEM was calculated for mean current traces and is expressed as a grey area fill above and below the average current trace. One-way analysis of variance (ANOVA) was used to compare more than two data sets, and a student's *t*-test was used to compare only two data sets. For data analyzed with ANOVA, a Dunnett post-hoc test was used. Differences were considered statistically significant at $p < 0.05$.

3. Results

$\alpha 6^*$ nAChRs are crucial for the rewarding properties of ethanol in rodents (Larsson et al., 2004; Lof et al., 2007; Powers et al., 2013). Systemic ethanol exposure is known to enhance AMPAR function on VTA DA neurons (Saal et al., 2003; Stuber et al., 2008), but $\alpha 6^*$ nAChR involvement in this process has not yet been studied. We began by testing the hypothesis that exposing slices to drinking-relevant concentrations of ethanol can increase AMPAR function on VTA DA neurons. VTA-containing brain slices were prepared from adult, drug-naïve mice, and slices were allowed to recover for 60 min. Slices were then incubated in ethanol or a control recording solution without ethanol for 60 min. After a washout period of 60 min (Fig. 1A), stable whole-cell recordings were established in VTA DA neurons. A second, AMPA-filled, micropipette was programmed to move adjacent to the recorded cell, pressure-eject (puff) AMPA (100 μ M), and be retracted (Engle et al., 2013). As previously described, the amplitude of AMPA-evoked currents at a holding potential of -60 mV was measured to assess AMPAR function (Engle et al., 2013). We found that incubating slices from non-Tg mice in ethanol (20 mM) for 60 min significantly increased AMPAR function in VTA DA neurons over baseline responses from control slices not exposed to ethanol (control = -187.3 ± 16 pA, 20 mM ethanol = -319.6 ± 43 pA; ANOVA, $p = 0.0034$; *post hoc* test, $p < 0.01$) (Fig. 1B, D, and E). To examine the role of $\alpha 6^*$ nAChRs, slices were pre-treated with α CtxMII (100 nM) for 10 min prior to ethanol incubation. Pretreatment with α CtxMII blocked ethanol-evoked increases in AMPAR

function in VTA DA neurons ($\alpha\text{CtxMII} = -216.7 \pm 44$ pA) (Fig. 1B and E), suggesting an important role for ongoing $\alpha 6^*$ nAChR activity in this process. αCtxMII by itself had no effect on AMPA-evoked currents (-182.6 ± 29 pA) (Fig. 1E). In a similar fashion, slices were pre-treated in tetrodotoxin (TTX) for 10 minutes prior to ethanol incubation to examine whether action potential firing during ethanol exposure is required to evoke an increase in AMPAR function. We found that TTX does not block ethanol-evoked increases in AMPAR function in VTA DA neurons (TTX = -342.3 ± 44 pA; $p < 0.05$) (Fig. 1E). To corroborate data suggesting that $\alpha 6^*$ nAChRs play a role in ethanol-mediated enhancement of AMPAR function, we studied changes in AMPAR function in slices from $\alpha 6\text{L9S}$ mice. Because these mice have enhanced $\alpha 6^*$ nAChR activity (Drenan et al., 2008), we hypothesized that a lower concentration of ethanol would be able to evoke increases in AMPAR function compared to non-Tg mice if $\alpha 6^*$ nAChRs are playing a role in this process. Baseline AMPAR function is not altered in $\alpha 6\text{L9S}$ mice. 5 mM ethanol was insufficient to alter AMPAR function in non-Tg slices (-171.0 ± 24 pA), but robustly enhanced AMPAR function in $\alpha 6\text{L9S}$ slices (control = -180.1 ± 22 pA, 5 mM ethanol = -416.1 ± 49 pA; ANOVA, $p < 0.0001$; *post hoc* test, $p < 0.0001$) (Fig. 1C, D, and F). This enhanced response was also blocked by pretreatment with αCtxMII (-223.0 ± 26 pA) (Fig. 1C and F), whereas αCtxMII alone had no effect (-192.3 ± 10 pA) (Fig. 1F). As in non-Tg mice, TTX blockade of action potential firing had no effect on ethanol's ability to enhance AMPAR function in $\alpha 6\text{L9S}$ mice (TTX = -365.9 ± 56 pA; $p < 0.01$) (Fig. 1F).

Enhanced $\alpha 6^*$ nAChR activity lowered the concentration of ethanol that is sufficient to enhance AMPAR function using a slice incubation procedure (Fig. 1C, D, and F). To determine whether the same trend occurred after *in vivo* exposure to ethanol, mice were administered intraperitoneal (i.p.) injections of ethanol. 60 min after this injection, mice were sacrificed and VTA brain slices were prepared. To minimize the possible effect of handling-induced stress, mice were handled once a day for three days, given a mock injection, and given a saline injection on the day immediately prior to the experiment (Fig. 2A). As in the previous experiment, AMPA was puff-applied onto a VTA DA neuron while recording inward currents evoked at a holding potential of -60 mV. Relative to a vehicle (saline) injection, ethanol (2.0 g/kg) significantly increased AMPAR function in VTA DA neurons in non-Tg slices (vehicle: -206.5 ± 24 pA, 2.0 g/kg ethanol: -365.2 ± 34 pA; ANOVA, $p = 0.0004$, *post hoc* test, $p < 0.001$) (Fig. 2B and D). A dose of 0.5 g/kg ethanol was insufficient to enhance AMPAR function in VTA DA neurons in non-Tg mice (-221.4 ± 17 pA) but did enhance AMPAR function in $\alpha 6\text{L9S}$ mice (vehicle: -197.5 ± 23 pA, 0.5 g/kg ethanol: -395.1 ± 46 pA; unpaired *t* test, $p = 0.0072$) (Fig. 2B–D). Together, these slice incubation and *in vivo* exposure experiments strongly indicate a role for $\alpha 6^*$ nAChRs in ethanol-mediated enhancement of AMPAR function in VTA.

Exposure of naïve brain slices to nicotine also enhances AMPAR function in VTA DA neurons (Engle et al., 2013; Jin et al., 2011; Mao et al., 2011). Although we previously demonstrated a role for $\alpha 6^*$ nAChRs in this process (Engle et al., 2013), we did not identify the nicotine concentration range over which this effect takes place. To examine the action of a combined incubation with nicotine and ethanol (Figs. 5 and 6), we first identified nicotine concentrations that were sufficient and insufficient to enhance AMPA-evoked currents on

VTA DA neurons. Brain slices cut from drug-naïve mice were incubated in nicotine for 60 min and after a 60 min wash-out period, AMPAR function was measured (Fig. 3A). In non-Tg brain slices, 100 nM nicotine was insufficient to increase AMPAR function, but 500 nM nicotine was sufficient (control = -187.3 ± 16 pA, 100 nM nicotine = -166.0 ± 14 , 500 nM nicotine = -283.4 ± 36 pA; ANOVA, $p = 0.0288$; *post hoc* test, $p < 0.05$) (Fig. 3B). In $\alpha 6$ L9S brain slices, nicotine concentrations as low as 10 nM are sufficient to increase AMPAR function (control = -180.1 ± 22 pA, 10 nM nicotine = -320.1 ± 41 pA; ANOVA, $p = 0.0012$; *post hoc* test, $p < 0.05$) (Fig. 3B). The highest concentration of nicotine tested that was insufficient to enhance AMPAR function in $\alpha 6$ L9S slices is 3 nM (-234.2 ± 36 pA) (Fig. 3B).

Our AMPA-evoked current approach measures AMPAR function from the total pool of surface-expressed AMPARs, including synaptic and extrasynaptic receptors. Because AMPARs are preferentially localized to synapses, we measured ethanol- and nicotine-elicited changes AMPA/NMDA ratios in VTA DA neurons using the same conditions we used for AMPA-evoked currents (Fig. 4A). AMPA to NMDA ratios were measured as described by McGehee and colleagues ((Mao et al., 2011); see Materials and Methods). As with AMPA-evoked currents, AMPA/NMDA ratios were significantly enhanced in non-Tg slices following exposure to either 20 mM ethanol or 500 nM nicotine (control = 2.47 ± 0.2 , 20 mM ethanol = 3.90 ± 0.1 , 500 nM nicotine = 3.41 ± 0.4 pA; ANOVA, $p = 0.0007$) (Fig. 4C). Lower concentrations of ethanol (5 mM) or nicotine (100 nM) did not increase AMPA/NMDA ratios (Fig. 4C), just as they did not increase AMPA-evoked current responses (Fig. 1D and 3B). Next, we measured AMPA/NMDA ratios in VTA DA neurons from $\alpha 6$ L9S slices incubated with either 5 mM ethanol or 100 nM nicotine – concentrations sufficient to enhance AMPA-evoked currents in these slices. Incubating $\alpha 6$ L9S slices for 60 min in either 5 mM ethanol or 100 nM nicotine was indeed sufficient to increase AMPA/NMDA ratios (control = 2.30 ± 0.2 , 5 mM ethanol = 4.13 ± 0.3 , 100 nM nicotine = 3.35 ± 0.2 ; ANOVA, $p = 0.0013$) (Fig. 4D). These results, together with our AMPA-evoked current data, suggest that $\alpha 6^*$ nAChR activity plays an important role mediating enhanced synaptic plasticity following nicotine and ethanol exposure.

Because both nicotine (Fig. 3) and ethanol (Fig. 1) act through $\alpha 6^*$ nAChRs to enhance AMPAR function in VTA DA neurons, we hypothesized that $\alpha 6^*$ nAChRs may also play a role in the combined action of nicotine+ethanol. To test this hypothesis, we incubated brain slices in control recording solution containing both nicotine and ethanol for 60 min and recorded AMPA-evoked currents after a 60 min washout period (Fig. 5A). Whereas 100 nM nicotine or 5 mM ethanol were insufficient to increase AMPAR function in non-Tg VTA DA neurons when incubated alone, simultaneous co-exposure to these concentrations enhanced AMPAR function (100 nM nicotine = -166.0 ± 14 pA, 5 mM ethanol = -171.0 ± 24 pA, 100 nM nicotine + 5 mM ethanol = -374.3 ± 69 pA; ANOVA, $p = 0.0080$) (Fig. 5B and C). To determine whether $\alpha 6^*$ nAChRs play a role in this response, slices were pre-incubated in α CtxMII (100 nM) for 10 min before adding nicotine and ethanol. α CtxMII blocked the combined action of nicotine+ethanol (-254.8 ± 39 pA) (Fig. 5C). Varenicline, a partial agonist at $\alpha 4\beta 2^*$ and $\alpha 6\beta 2^*$ nAChRs (Grady et al., 2010), is currently used as a smoking cessation drug. To determine whether varenicline interferes with the combined

action of nicotine+ethanol, slices were pre-incubated in varenicline for 10 min followed by continuous co-incubation of slices in varenicline (1 μ M), nicotine (100 nM), and ethanol (5 mM). Like α CtxMII, varenicline blocked increases in AMPAR function mediated by nicotine+ethanol co-exposure (-227.8 ± 41 pA) (Fig. 5C). Varenicline by itself had no effect on AMPAR function in non-Tg cells (-204.3 ± 25 pA) (Fig. 5C).

To further test the idea that $\alpha 6^*$ nAChRs play a role in AMPAR enhancement following nicotine+ethanol co-exposure, we performed an analogous set of experiments with $\alpha 6$ L9S VTA slices (Fig. 6A). We previously determined that 3 nM nicotine (Fig. 3B) and 200 μ M ethanol (Fig. 1D) were each insufficient to enhance AMPAR function in $\alpha 6$ L9S slices when administered alone. However, when co-administered to $\alpha 6$ L9S slices, 3 nM nicotine and 200 μ M ethanol enhanced AMPAR function (200 μ M ethanol = -217.8 ± 49 pA, 3 nM nicotine = -234.2 ± 36 pA, 200 μ M ethanol + 3 nM nicotine = -442.1 ± 72 pA; ANOVA, $p = 0.0065$) (Fig. 6B and C). As with non-Tg slices, pre-treatment with either α CtxMII or varenicline blocked the enhancement in AMPAR function seen following nicotine+ethanol co-exposure (MII: -248.0 ± 25 pA, varenicline: -252.0 ± 35 pA) (Fig. 6C). As in non-Tg slices, varenicline alone did not alter AMPAR function in $\alpha 6$ L9S slices (-246.5 ± 28 pA) (Fig. 6C).

4. Discussion

In this paper, we used converging pharmacological and genetic approaches to study the action of nAChRs in drug-mediated enhancement of AMPAR function and synaptic plasticity in VTA DA neurons. Our results indicate that $\alpha 6^*$ nAChR activity is important in ethanol-mediated enhancement of AMPAR function, and *in vivo* drug exposure experiments corroborated data acquired using *in vitro* methods. $\alpha 6^*$ nAChRs are also critical for the ability of combined, subthreshold nicotine+ethanol concentrations to enhance AMPAR function. Finally, we show that varenicline, an FDA-approved smoking cessation compound, is capable of disrupting the activity of nicotine+ethanol co-exposure at VTA DA neurons.

4.1 Ethanol-mediated enhancement of AMPAR function: Mechanisms

Ungless and colleagues demonstrated that a single cocaine exposure produced enhanced AMPAR function on the surface of VTA DA neurons (Ungless et al., 2001). This was later appreciated to be a generalized effect of exposure to drugs of abuse, as a single exposure to nicotine, ethanol, or other drugs produced the same effect (Saal et al., 2003). Voluntary drug exposure also enhances AMPAR function (Stuber et al., 2008), and enhanced glutamate receptor function plays a critical role in controlling the persistence of subsequent drug taking (Cannady et al., 2013; Engblom et al., 2008). $\alpha 6^*$ nAChR activity plays a key role in ethanol consumption, as blockade of these receptors in VTA with α CtxMII attenuated ethanol-stimulated locomotion, DA release, and conditioned responding (Larsson et al., 2004; Lof et al., 2007). Together with Chester and colleagues, we recently demonstrated that $\alpha 6$ L9S mice show increased sensitivity to ethanol in conditioned place preference experiments and 2-bottle choice drinking assays, further supporting a role for $\alpha 6^*$ nAChRs in ethanol's action in the VTA (Powers et al., 2013). We also demonstrated that selective activation of $\alpha 6^*$ nAChRs by nicotine 1) enhances AMPAR function in VTA neurons

(Engle et al., 2013), and 2) can condition a place preference to nicotine (Drenan et al., 2012). Thus, our data in this report showing that $\alpha 6^*$ nAChR activity plays an important role in enhanced glutamatergic transmission in VTA is consistent with previous findings.

Ethanol may act directly on nAChRs on the surface of VTA neurons to produce enhanced AMPAR activity, but this activity is not likely to be analogous to a classical orthosteric agonist. Similar to ethanol's ability to potentiate GABA_A receptor activity (Mehta and Ticku, 1988), evidence points more toward ethanol-mediated potentiation of nAChR activity. Ethanol enhanced ACh-elicited inward currents for several nAChR subtypes expressed in *Xenopus* oocytes, including $\alpha 4\beta 2$ nAChRs (Cardoso et al., 1999). Single channel recordings from cells expressing cloned $\alpha 4\beta 2$ nAChRs indicates that ethanol enhances ACh-induced currents by stabilizing the open state of the receptor (Zuo et al., 2004). ACh, presumably released from mesopontine axon terminals, is present in VTA slices and contributes to baseline $\alpha 6^*$ nAChR activity in VTA DA neurons (Drenan et al., 2008). Our demonstration that a $\alpha 6^*$ nAChR antagonist blocks ethanol-mediated enhancement of AMPAR function (Fig. 1) suggests that endogenous ACh and ethanol combine at $\alpha 6^*$ nAChRs to produce sufficient nAChR activity to initiate signaling events ultimately resulting in AMPAR functional enhancement. Our previous reports (Drenan et al., 2010; Engle et al., 2013), and one from the laboratory of Tapper and colleagues (Liu et al., 2012), suggest that $\alpha 4$ nAChR subunits are incorporated into $\alpha 6^*$ nAChRs that mediate these effects. Thus, most VTA $\alpha 6^*$ nAChRs contain $\alpha 4$ and $\beta 2$ subunits and could thus be subject to ethanol's nAChR-potentiating properties.

Ethanol is known to alter DA neuron activity and accumbal DA release via indirect, activity-dependent mechanisms. For example, ethanol increases ACh release in VTA (Larsson et al., 2005), and ACh-induced firing in VTA DA neurons can be potentiated by ethanol (Liu et al., 2013). Ethanol may also contribute to reward by reducing GABAergic inhibition to VTA DA neurons (Di Chiara et al., 1996; Spanagel et al., 1992). Although it is possible that ethanol and nAChR activation collaborate to enhance AMPAR function via either of these mechanisms, our data showing that neuronal activity is not required to enhance AMPAR function after ethanol exposure (Fig. 1E and F) more strongly support a direct mechanism of action on DA neurons. Indeed, ACh that is released in VTA slices and which contributes to baseline $\alpha 6^*$ nAChR activity is insensitive to TTX (Drenan et al., 2008). Recently, Berg and colleagues reported a novel, cell-autonomous mechanism by which nicotine elicits enhanced AMPAR trafficking to the surface of hippocampal neurons (Halff et al., 2014). Our data showing that AMPAR function is enhanced even in the presence of TTX (Fig. 1) suggest that a similar cell-autonomous, $\alpha 6$ -mediated effect could also occur in VTA DA neurons.

4.2 The combined activity of nicotine and ethanol in enhancement of AMPAR function

Our data demonstrating that subthreshold concentrations of nicotine and ethanol can combine to enhance AMPAR function on the surface of VTA DA neurons is consistent with previous reports utilizing simultaneous nicotine+ethanol co-exposure. In brain slice experiments examining drug-stimulated action potential firing in VTA DA neurons, subthreshold concentrations of nicotine were able to synergize with ethanol to enhance firing rate (Clark and Little, 2004). Similarly, intra-VTA infusion of subthreshold nicotine

combined with intraperitoneal injection of subthreshold ethanol resulted in enhanced accumbal DA release relative to nicotine alone or ethanol alone (Tizabi et al., 2002). In behavioral experiments, nicotine+ethanol co-exposure produced greater stimulation of locomotor activity compared to activity produced by nicotine alone or ethanol alone (Schaefer and Michael, 1992).

Our demonstration that 100 nM nicotine plus 5 mM ethanol combine to enhance VTA AMPAR function is relevant to human consumption of these drugs. A blood alcohol concentration of approximately 25 mg/dL (or 5 mM) is achievable with one drink and would not be expected to produce behavioral changes in humans other than mild euphoria and/or slight social disinhibition. A nicotine plasma level of 100 nM is easily achievable by one cigarette in a person naïve to nicotine (Henningfield, 1995). In a daily smoker, this level of nicotine in plasma would be attainable by afternoon or evening simply due to accumulation of the drug from cigarettes smoked throughout the day (DHHS, 1988). These properties of nicotine and ethanol, taken together with our data suggest that, in humans, one drink plus one cigarette may be sufficient to produce plastic changes in VTA DA neurons that are relevant to the addiction process.

Genetic evidence points to a prominent role for $\alpha 6$ -containing nAChRs in alcohol consumption as well as nicotine addiction (Hoft et al., 2009a; Hoft et al., 2009b). Our data suggest that $\alpha 6$ -containing nAChRs play a central role in the response to subthreshold, co-applied concentrations of nicotine and ethanol. In experiments involving DA release from striatal synaptosomes, α CtxMII-sensitive ($\alpha 6$ -dependent) nAChRs are the most sensitive to nicotine (Salminen et al., 2007), supporting the notion that they are capable of responding meaningfully to subthreshold nicotine when ethanol is present to enhance receptor activity. In further support of the idea that $\alpha 6$ -containing nAChRs confer the ability to respond to very low concentrations of nicotine, $\alpha 6$ KO mice have reduced ability to self-administer low (10 ng) concentrations of nicotine (Exley et al., 2011).

Varenicline is a partial agonist with similar efficacy and potency at both α CtxMII-sensitive ($\alpha 6$ -containing) and α CtxMII-resistant (non- $\alpha 6$) nAChRs (Grady et al., 2010). Varenicline is thought to promote abstinence from smoking via a dual mechanism: 1) varenicline substitutes for nicotine in activating nAChRs, and 2) varenicline competes with and blunts the full action of nicotine in activating nAChRs (Rollema et al., 2007). Our results suggest that varenicline, similar to α CtxMII, may interfere with the ability of nicotine and/or ethanol to enhance AMPAR function in VTA neurons. This is consistent with varenicline's action in preclinical and clinical studies. For example, varenicline reduces ethanol intake in mice (Hendrickson et al., 2010) and rats (Steensland et al., 2007). Varenicline also reduces alcohol consumption in heavy drinking smokers (Fucito et al., 2011; McKee et al., 2009; Mitchell et al., 2012). Together, this suggests that persons taking varenicline may be afforded some protection from the addictive properties of subthreshold concentrations of nicotine+alcohol. Varenicline, or other compounds with partial agonist properties at $\alpha 4^*$ and/or $\alpha 6^*$ nAChRs but a better side-effect profile, could be useful in attenuating nicotine/alcohol co-addiction.

4.3 Conclusions

This paper provides new details on the mechanistic underpinnings of nicotine+ethanol co-addiction. Nicotine and ethanol cooperate and/or synergize to enhance excitatory transmission in the mesolimbic dopamine pathway, the brain's key reward circuit. nAChRs containing $\alpha 6$ subunits are important players in this response, and varenicline or other nAChR partial agonists may prove useful for attenuating the combined action of nicotine +ethanol.

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References

- Argilli E, Sibley DR, Malenka RC, England PM, Bonci A. Mechanism and time course of cocaine-induced long-term potentiation in the ventral tegmental area. *J Neurosci.* 2008; 28:9092–9100. 28/37/9092 [pii]. 10.1523/JNEUROSCI.1001-08.2008 [PubMed: 18784289]
- Azam L, Maskos U, Changeux JP, Dowell CD, Christensen S, De Biasi M, McIntosh JM. α -Conotoxin BuIA[T5A;P6O]: a novel ligand that discriminates between $\alpha 6\beta 4$ and $\alpha 6\beta 2$ nicotinic acetylcholine receptors and blocks nicotine-stimulated norepinephrine release. *FASEB J.* 2010; 24:5113–5123. fj. 10-166272 [pii]. 10.1096/fj.10-166272 [PubMed: 20739611]
- Bobo JK. Nicotine dependence and alcoholism epidemiology and treatment. *J Psychoactive Drugs.* 1992; 24:123–129. [PubMed: 1506997]
- Borgland SL, Malenka RC, Bonci A. Acute and chronic cocaine-induced potentiation of synaptic strength in the ventral tegmental area: electrophysiological and behavioral correlates in individual rats. *J Neurosci.* 2004; 24:7482–7490. 24/34/7482 [pii]. 10.1523/JNEUROSCI.1312-04.2004 [PubMed: 15329395]
- Calabresi P, Lacey MG, North RA. Nicotinic excitation of rat ventral tegmental neurones in vitro studied by intracellular recording. *Br J Pharmacol.* 1989; 98:135–140. [PubMed: 2804543]
- Cannady R, Fisher KR, Durant B, Besheer J, Hodge CW. Enhanced AMPA receptor activity increases operant alcohol self-administration and cue-induced reinstatement. *Addict Biol.* 2013; 18:54–65. 10.1111/adb.12000 [PubMed: 23126443]
- Cardoso RA, Brozowski SJ, Chavez-Noriega LE, Harpold M, Valenzuela CF, Harris RA. Effects of ethanol on recombinant human neuronal nicotinic acetylcholine receptors expressed in *Xenopus* oocytes. *J Pharmacol Exp Ther.* 1999; 289:774–780. [PubMed: 10215652]
- Champtiaux N, Gotti C, Cordero-Erausquin M, David DJ, Przybylski C, Lena C, Clementi F, Moretti M, Rossi FM, Le Novère N, McIntosh JM, Gardier AM, Changeux JP. Subunit composition of functional nicotinic receptors in dopaminergic neurons investigated with knock-out mice. *J Neurosci.* 2003; 23:7820–7829. [PubMed: 12944511]
- Clark A, Little HJ. Interactions between low concentrations of ethanol and nicotine on firing rate of ventral tegmental dopamine neurones. *Drug Alcohol Depend.* 2004; 75:199–206. S0376871604000663 [pii]. 10.1016/j.drugalcdep.2004.03.001 [PubMed: 15276226]
- Cohen BN, Mackey ED, Grady SR, McKinney S, Patzlaff NE, Wageman CR, McIntosh JM, Marks MJ, Lester HA, Drenan RM. Nicotinic cholinergic mechanisms causing elevated dopamine release and abnormal locomotor behavior. *Neuroscience.* 2012; 200:31–41. S0306-4522(11)01240-1 [pii]. 10.1016/j.neuroscience.2011.10.047 [PubMed: 22079576]
- Creed MC, Ntamaty NR, Tan KR. VTA GABA neurons modulate specific learning behaviors through the control of dopamine and cholinergic systems. *Front Behav Neurosci.* 2014; 8:8.10.3389/fnbeh.2014.00008 [PubMed: 24478655]

- DHHS. The health consequences of smoking: nicotine addiction: a report of the Surgeon General. Washington, D.C.: 1988.
- Di Chiara G, Acquas E, Tanda G. Ethanol as a neurochemical surrogate of conventional reinforcers: the dopamine-opioid link. *Alcohol*. 1996; 13:13–17. [PubMed: 8837928]
- DiFranza JR, Guerrera MP. Alcoholism and smoking. *J Stud Alcohol*. 1990; 51:130–135. [PubMed: 2308350]
- Dong Y, Saal D, Thomas M, Faust R, Bonci A, Robinson T, Malenka RC. Cocaine-induced potentiation of synaptic strength in dopamine neurons: behavioral correlates in GluRA(–/–) mice. *Proc Natl Acad Sci U S A*. 2004; 101:14282–14287. 0401553101 [pii]. 10.1073/pnas.0401553101 [PubMed: 15375209]
- Doyon WM, Dong Y, Ostroumov A, Thomas AM, Zhang TA, Dani JA. Nicotine decreases ethanol-induced dopamine signaling and increases self-administration via stress hormones. *Neuron*. 2013; 79:530–540.10.1016/j.neuron.2013.06.006 [PubMed: 23871233]
- Drenan, RM.; Engle, SE.; Lester, HA.; McIntosh, JM.; Brunzell, DH. Program No. 455.03/P19. 2012 Neuroscience Meeting Planner. New Orleans, LA: Society for Neuroscience; 2012. Activation of $\alpha 6 \beta 2^*$ nicotinic acetylcholine receptors is sufficient for nicotine reward. Online
- Drenan RM, Grady SR, Steele AD, McKinney S, Patzlaff NE, McIntosh JM, Marks MJ, Miwa JM, Lester HA. Cholinergic modulation of locomotion and striatal dopamine release is mediated by $\alpha 6 \alpha 4^*$ nicotinic acetylcholine receptors. *J Neurosci*. 2010; 30:9877–9889. [PubMed: 20660270]
- Drenan RM, Grady SR, Whiteaker P, McClure-Begley T, McKinney S, Miwa JM, Bupp S, Heintz N, McIntosh JM, Bencherif M, Marks MJ, Lester HA. *In vivo* activation of midbrain dopamine neurons via sensitized, high-affinity $\alpha 6^*$ nicotinic acetylcholine receptors. *Neuron*. 2008; 60:123–136. [PubMed: 18940593]
- Engblom D, Bilbao A, Sanchis-Segura C, Dahan L, Perreau-Lenz S, Balland B, Parkitna JR, Lujan R, Halbout B, Mameli M, Parlato R, Sprengel R, Luscher C, Schutz G, Spanagel R. Glutamate receptors on dopamine neurons control the persistence of cocaine seeking. *Neuron*. 2008; 59:497–508. S0896-6273(08)00582-5 [pii]. 10.1016/j.neuron.2008.07.010 [PubMed: 18701074]
- Engle SE, Broderick HJ, Drenan RM. Local application of drugs to study nicotinic acetylcholine receptor function in mouse brain slices. *J Vis Exp*. 2012:e50034. [pii]. 10.3791/5003450034 [PubMed: 23128482]
- Engle SE, Shih PY, McIntosh JM, Drenan RM. $\alpha 4 \alpha 6 \beta 2^*$ nicotinic acetylcholine receptor activation on ventral tegmental area dopamine neurons is sufficient to stimulate a depolarizing conductance and enhance surface AMPA receptor function. *Mol Pharmacol*. 2013; 84:393–406.10.1124/mol.113.087346 [PubMed: 23788655]
- Etherton MR, Tabuchi K, Sharma M, Ko J, Sudhof TC. An autism-associated point mutation in the neuroligin cytoplasmic tail selectively impairs AMPA receptor-mediated synaptic transmission in hippocampus. *EMBO J*. 2011; 30:2908–2919.10.1038/emboj.2011.182 [PubMed: 21642956]
- Exley R, Maubourguet N, David V, Eddine R, Evrard A, Pons S, Marti F, Threlfell S, Cazala P, McIntosh JM, Changeux JP, Maskos U, Cragg SJ, Faure P. Distinct contributions of nicotinic acetylcholine receptor subunit $\alpha 4$ and subunit $\alpha 6$ to the reinforcing effects of nicotine. *Proc Natl Acad Sci U S A*. 2011; 108:7577–7582. 1103000108 [pii]. 10.1073/pnas.1103000108 [PubMed: 21502501]
- Fucito LM, Toll BA, Wu R, Romano DM, Tek E, O'Malley SS. A preliminary investigation of varenicline for heavy drinking smokers. *Psychopharmacology (Berl)*. 2011; 215:655–663.10.1007/s00213-010-2160-9 [PubMed: 21221531]
- Gao M, Jin Y, Yang K, Zhang D, Lukas RJ, Wu J. Mechanisms involved in systemic nicotine-induced glutamatergic synaptic plasticity on dopamine neurons in the ventral tegmental area. *J Neurosci*. 2010; 30:13814–13825. 30/41/13814 [pii]. 10.1523/JNEUROSCI.1943-10.2010 [PubMed: 20943922]
- Grady SR, Drenan RM, Breining SR, Yohannes D, Wageman CR, Fedorov NB, McKinney S, Whiteaker P, Bencherif M, Lester HA, Marks MJ. Structural differences determine the relative selectivity of nicotinic compounds for native $\alpha 4 \beta 2^*$ -, $\alpha 6 \beta 2^*$ -, $\alpha 3 \beta 4^*$ - and $\alpha 7$ -nicotine acetylcholine receptors. *Neuropharmacology*. 2010; 58:1054–1066. [PubMed: 20114055]

- Grant BF, Hasin DS, Chou SP, Stinson FS, Dawson DA. Nicotine dependence and psychiatric disorders in the United States: results from the national epidemiologic survey on alcohol and related conditions. *Arch Gen Psychiatry*. 2004; 61:1107–1115. 61/11/1107 [pii]. 10.1001/archpsyc.61.11.1107 [PubMed: 15520358]
- Halff AW, Gomez-Varela D, John D, Berg DK. A novel mechanism for nicotinic potentiation of glutamatergic synapses. *J Neurosci*. 2014; 34:2051–2064.10.1523/JNEUROSCI.2795-13.2014 [PubMed: 24501347]
- Harrison EL, Hinson RE, McKee SA. Experimenting and daily smokers: episodic patterns of alcohol and cigarette use. *Addict Behav*. 2009; 34:484–486.10.1016/j.addbeh.2008.12.013 [PubMed: 19176271]
- Hendrickson LM, Zhao-Shea R, Pang X, Gardner PD, Tapper AR. Activation of $\alpha 4^*$ nAChRs is necessary and sufficient for varenicline-induced reduction of alcohol consumption. *J Neurosci*. 2010; 30:10169–10176. 30/30/10169 [pii]. 10.1523/JNEUROSCI.2601-10.2010 [PubMed: 20668200]
- Henningfield JE. Nicotine medications for smoking cessation. *N Engl J Med*. 1995; 333:1196–1203.10.1056/NEJM199511023331807 [PubMed: 7565976]
- Hnasko TS, Edwards RH. Neurotransmitter corelease: mechanism and physiological role. *Annu Rev Physiol*. 2012; 74:225–243.10.1146/annurev-physiol-020911-153315 [PubMed: 22054239]
- Hnasko TS, Hjelmstad GO, Fields HL, Edwards RH. Ventral tegmental area glutamate neurons: electrophysiological properties and projections. *J Neurosci*. 2012; 32:15076–15085.10.1523/JNEUROSCI.3128-12.2012 [PubMed: 23100428]
- Hoft NR, Corley RP, McQueen MB, Huizinga D, Menard S, Ehringer MA. SNPs in CHRNA6 and CHRNA3 are associated with alcohol consumption in a nationally representative sample. *Genes Brain Behav*. 2009a; 8:631–637. GBB495 [pii]. 10.1111/j.1601-183X.2009.00495.x [PubMed: 19500157]
- Hoft NR, Corley RP, McQueen MB, Schlaepfer IR, Huizinga D, Ehringer MA. Genetic association of the CHRNA6 and CHRNA3 genes with tobacco dependence in a nationally representative sample. *Neuropsychopharmacology*. 2009b; 34:698–706.10.1038/npp.2008.122 [PubMed: 18704094]
- Jin Y, Yang K, Wang H, Wu J. Exposure of nicotine to ventral tegmental area slices induces glutamatergic synaptic plasticity on dopamine neurons. *Synapse*. 2011; 65:332–338.10.1002/syn.20850 [PubMed: 20730803]
- Kauer JA, Malenka RC. Synaptic plasticity and addiction. *Nat Rev Neurosci*. 2007; 8:844–858. nrm2234 [pii]. 10.1038/nrm2234 [PubMed: 17948030]
- King AC, Epstein AM. Alcohol dose-dependent increases in smoking urge in light smokers. *Alcohol Clin Exp Res*. 2005; 29:547–552. [PubMed: 15834219]
- Lammel S, Hetzel A, Hackel O, Jones I, Liss B, Roeper J. Unique properties of mesoprefrontal neurons within a dual mesocorticolimbic dopamine system. *Neuron*. 2008; 57:760–773. [PubMed: 18341995]
- Larsson A, Edstrom L, Svensson L, Soderpalm B, Engel JA. Voluntary ethanol intake increases extracellular acetylcholine levels in the ventral tegmental area in the rat. *Alcohol Alcohol*. 2005; 40:349–358.10.1093/alcalc/agh180 [PubMed: 16043436]
- Larsson A, Jerlhag E, Svensson L, Soderpalm B, Engel JA. Is an α -conotoxin MII-sensitive mechanism involved in the neurochemical, stimulatory, and rewarding effects of ethanol? *Alcohol*. 2004; 34:239–250. [PubMed: 15902919]
- le Novere N, Zoli M, Lena C, Ferrari R, Picciotto MR, Merlo-Pich E, Changeux JP. Involvement of $\alpha 6$ nicotinic receptor subunit in nicotine-elicited locomotion, demonstrated by in vivo antisense oligonucleotide infusion. *Neuroreport*. 1999; 10:2497–2501. [PubMed: 10574359]
- Lena C, de Kerchove D'Exaerde A, Cordero-Erausquin M, Le Novere N, del Mar Arroyo-Jimenez M, Changeux JP. Diversity and distribution of nicotinic acetylcholine receptors in the locus ceruleus neurons. *Proc Natl Acad Sci U S A*. 1999; 96:12126–12131. [PubMed: 10518587]
- Liu L, Hendrickson LM, Guildford MJ, Zhao-Shea R, Gardner PD, Tapper AR. Nicotinic acetylcholine receptors containing the $\alpha 4$ subunit modulate alcohol reward. *Biol Psychiatry*. 2013; 73:738–746.10.1016/j.biopsych.2012.09.019 [PubMed: 23141806]

- Liu L, Zhao-Shea R, McIntosh JM, Gardner P, Tapper A. Nicotine Persistently Activates Ventral Tegmental Area Dopaminergic Neurons Via Nicotinic Acetylcholine Receptors Containing $\alpha 4$ and $\alpha 6$ subunits. *Mol Pharmacol*. 2012 mol.111.076661 [pii]. 10.1124/mol.111.076661
- Lof E, Olausson P, deBejczy A, Stomberg R, McIntosh JM, Taylor JR, Soderpalm B. Nicotinic acetylcholine receptors in the ventral tegmental area mediate the dopamine activating and reinforcing properties of ethanol cues. *Psychopharmacology (Berl)*. 2007; 195:333–343.10.1007/s00213-007-0899-4 [PubMed: 17703283]
- Luscher C, Malenka RC. Drug-evoked synaptic plasticity in addiction: from molecular changes to circuit remodeling. *Neuron*. 2011; 69:650–663. S0896-6273(11)00065-1 [pii]. 10.1016/j.neuron.2011.01.017 [PubMed: 21338877]
- Mackey ED, Engle SE, Kim MR, O'Neill HC, Wageman CR, Patzlaff NE, Wang Y, Grady SR, McIntosh JM, Marks MJ, Lester HA, Drenan RM. $\alpha 6^*$ Nicotinic Acetylcholine Receptor Expression and Function in a Visual Salience Circuit. *J Neurosci*. 2012; 32:10226–10237. 32/30/10226 [pii]. 10.1523/JNEUROSCI.0007-12.2012 [PubMed: 22836257]
- Mao D, Gallagher K, McGehee DS. Nicotine potentiation of excitatory inputs to ventral tegmental area dopamine neurons. *J Neurosci*. 2011; 31:6710–6720. 31/18/6710 [pii]. 10.1523/JNEUROSCI.5671-10.2011 [PubMed: 21543600]
- Marubio LM, Gardier AM, Durier S, David D, Klink R, Arroyo-Jimenez MM, McIntosh JM, Rossi F, Champniaux N, Zoli M, Changeux JP. Effects of nicotine in the dopaminergic system of mice lacking the $\alpha 4$ subunit of neuronal nicotinic acetylcholine receptors. *Eur J Neurosci*. 2003; 17:1329–1337. [PubMed: 12713636]
- McKee SA, Harrison EL, O'Malley SS, Krishnan-Sarin S, Shi J, Tetrault JM, Picciotto MR, Petrakis IL, Estevez N, Balchunas E. Varenicline reduces alcohol self-administration in heavy-drinking smokers. *Biol Psychiatry*. 2009; 66:185–190.10.1016/j.biopsych.2009.01.029 [PubMed: 19249750]
- Mehta AK, Ticku MK. Ethanol potentiation of GABAergic transmission in cultured spinal cord neurons involves gamma-aminobutyric acidA-gated chloride channels. *J Pharmacol Exp Ther*. 1988; 246:558–564. [PubMed: 2457076]
- Miller NS, Gold MS. Comorbid cigarette and alcohol addiction: epidemiology and treatment. *J Addict Dis*. 1998; 17:55–66.10.1300/J069v17n01_06 [PubMed: 9549603]
- Mitchell JM, Teague CH, Kayser AS, Bartlett SE, Fields HL. Varenicline decreases alcohol consumption in heavy-drinking smokers. *Psychopharmacology (Berl)*. 2012; 223:299–306.10.1007/s00213-012-2717-x [PubMed: 22547331]
- Mitchell SH, de Wit H, Zacny JP. Effects of varying ethanol dose on cigarette consumption in healthy normal volunteers. *Behav Pharmacol*. 1995; 6:359–365. [PubMed: 11224344]
- Nashmi R, Xiao C, Deshpande P, McKinney S, Grady SR, Whiteaker P, Huang Q, McClure-Begley T, Lindstrom JM, Labarca C, Collins AC, Marks MJ, Lester HA. Chronic nicotine cell specifically upregulates functional $\alpha 4^*$ nicotinic receptors: basis for both tolerance in midbrain and enhanced long-term potentiation in perforant path. *J Neurosci*. 2007; 27:8202–8218. [PubMed: 17670967]
- Pons S, Fattore L, Cossu G, Tolu S, Porcu E, McIntosh JM, Changeux JP, Maskos U, Fratta W. Crucial role of $\alpha 4$ and $\alpha 6$ nicotinic acetylcholine receptor subunits from ventral tegmental area in systemic nicotine self-administration. *J Neurosci*. 2008; 28:12318–12327. [PubMed: 19020025]
- Powers MS, Broderick HJ, Drenan RM, Chester JA. Nicotinic acetylcholine receptors containing $\alpha 6$ subunits contribute to alcohol reward-related behaviours. *Genes Brain Behav*. 2013; 12:543–553.10.1111/gbb.12042 [PubMed: 23594044]
- Rollema H, Chambers LK, Coe JW, Glowa J, Hurst RS, Lebel LA, Lu Y, Mansbach RS, Mather RJ, Rovetti CC, Sands SB, Schaeffer E, Schulz DW, Tingley FD 3rd, Williams KE. Pharmacological profile of the $\alpha 4\beta 2$ nicotinic acetylcholine receptor partial agonist varenicline, an effective smoking cessation aid. *Neuropharmacology*. 2007; 52:985–994.10.1016/j.neuropharm.2006.10.016 [PubMed: 17157884]
- Rose JE, Brauer LH, Behm FM, Cramblett M, Calkins K, Lawhon D. Psychopharmacological interactions between nicotine and ethanol. *Nicotine Tob Res*. 2004; 6:133–144.10.1080/14622200310001656957 [PubMed: 14982697]

- Ross SA, Wong JY, Clifford JJ, Kinsella A, Massalas JS, Horne MK, Scheffer IE, Kola I, Waddington JL, Berkovic SF, Drago J. Phenotypic characterization of an $\alpha 4$ neuronal nicotinic acetylcholine receptor subunit knock-out mouse. *J Neurosci*. 2000; 20:6431–6441. [PubMed: 10964949]
- Saal D, Dong Y, Bonci A, Malenka RC. Drugs of abuse and stress trigger a common synaptic adaptation in dopamine neurons. *Neuron*. 2003; 37:577–582. S0896627303000217 [pii]. [PubMed: 12597856]
- Salminen O, Drapeau JA, McIntosh JM, Collins AC, Marks MJ, Grady SR. Pharmacology of α -Conotoxin MII-Sensitive Subtypes of Nicotinic Acetylcholine Receptors Isolated by Breeding of Null Mutant Mice. *Mol Pharmacol*. 2007; 71:1563–1571. [PubMed: 17341654]
- Salminen O, Murphy KL, McIntosh JM, Drago J, Marks MJ, Collins AC, Grady SR. Subunit composition and pharmacology of two classes of striatal presynaptic nicotinic acetylcholine receptors mediating dopamine release in mice. *Mol Pharmacol*. 2004; 65:1526–1535. [PubMed: 15155845]
- Schaefer GJ, Michael RP. Interactions between alcohol and nicotine on intracranial self-stimulation and locomotor activity in rats. *Drug Alcohol Depend*. 1992; 30:37–47. [PubMed: 1591979]
- Spanagel R, Herz A, Shippenberg TS. Opposing tonically active endogenous opioid systems modulate the mesolimbic dopaminergic pathway. *Proc Natl Acad Sci U S A*. 1992; 89:2046–2050. [PubMed: 1347943]
- Steensland P, Simms JA, Holgate J, Richards JK, Bartlett SE. Varenicline, an $\alpha 4\beta 2$ nicotinic acetylcholine receptor partial agonist, selectively decreases ethanol consumption and seeking. *Proc Natl Acad Sci U S A*. 2007; 104:12518–12523. 0705368104 [pii]. 10.1073/pnas.0705368104 [PubMed: 17626178]
- Stuber GD, Hopf FW, Hahn J, Cho SL, Guillory A, Bonci A. Voluntary ethanol intake enhances excitatory synaptic strength in the ventral tegmental area. *Alcohol Clin Exp Res*. 2008; 32:1714–1720. 10.1111/j.1530-0277.2008.00749.x [PubMed: 18627359]
- Sulzer D, Joyce MP, Lin L, Geldwert D, Haber SN, Hattori T, Rayport S. Dopamine neurons make glutamatergic synapses in vitro. *J Neurosci*. 1998; 18:4588–4602. [PubMed: 9614234]
- Tapper AR, McKinney SL, Nashmi R, Schwarz J, Deshpande P, Labarca C, Whiteaker P, Marks MJ, Collins AC, Lester HA. Nicotine activation of $\alpha 4^*$ receptors: sufficient for reward, tolerance, and sensitization. *Science*. 2004; 306:1029–1032. [PubMed: 15528443]
- Tizabi Y, Copeland RL Jr, Louis VA, Taylor RE. Effects of combined systemic alcohol and central nicotine administration into ventral tegmental area on dopamine release in the nucleus accumbens. *Alcohol Clin Exp Res*. 2002; 26:394–399. [PubMed: 11923594]
- Ungless MA, Whistler JL, Malenka RC, Bonci A. Single cocaine exposure in vivo induces long-term potentiation in dopamine neurons. *Nature*. 2001; 411:583–587. 35079077 [pii]. 10.1038/35079077 [PubMed: 11385572]
- Whiteaker P, McIntosh JM, Luo S, Collins AC, Marks MJ. [125 I]- α -Conotoxin MII identifies a novel nicotinic acetylcholine receptor population in mouse brain. *Mol Pharmacol*. 2000; 57:913–925. [PubMed: 10779374]
- WHO. Department of Mental Health and Substance Abuse, Global Status Report on Alcohol 2011. World Health Organization Department of Mental Health and Substance Abuse; Geneva: 2011.
- Zuo Y, Nagata K, Yeh JZ, Narahashi T. Single-channel analyses of ethanol modulation of neuronal nicotinic acetylcholine receptors. *Alcohol Clin Exp Res*. 2004; 28:688–696. 00000374-200405000-00002 [pii]. [PubMed: 15166642]

Highlights

1. Ethanol enhances AMPA receptor function in VTA DA neurons via $\alpha 6^*$ nAChRs.
2. Ethanol-mediated increases in AMPAR function are action potential independent.
3. Combining subthreshold concentrations of nicotine and ethanol enhance AMPAR function.
4. Varenicline blocks enhanced AMPAR function mediated by nicotine+ethanol co-exposure.

Figure 1.

$\alpha 6^*$ nAChRs are involved in ethanol-mediated increases in AMPAR function in VTA DA neurons. (A) Schematic illustrating ethanol exposure after cutting brain slices containing the VTA. After a 60 min recovery period, brain slices were incubated in a control holding solution or an ethanol-containing holding solution for 60 min. Slices were transferred out of ethanol for a 60 min washout period before initiating whole-cell recordings from VTA DA neurons. An additional group of brain slices were incubated in an inhibitor (inh), either 100 nM α CtxMII or 0.5 μ M TTX, for 10 min before starting the 60 min ethanol incubation. (B and C) Average (black trace) AMPA-evoked current traces from VTA DA neurons in (B) non-Tg and (C) $\alpha 6$ L9S brain slices incubated in control recording solution or ethanol. Grey area fill indicates SEM for the average current trace. AMPA (100 μ M) was puff-applied while holding VTA DA neurons at -60 mV. Scale: 100 pA, 5 sec. (D) Summary of AMPA-evoked responses (mean $\pm$ SEM) from VTA DA neurons at a holding potential of -60 mV in $\alpha 6$ L9S mice and their non-Tg littermates. Dotted line indicates average amplitude of baseline AMPA-evoked responses. (E and F) Summary of AMPA-evoked current amplitudes in VTA DA neurons in (E) non-Tg and (F) $\alpha 6$ L9S brain slices. Data are shown as follows: grey boxes enclose the inner (25% to 75%) quartiles with the middle line

indicating the median, and whiskers indicate the maximum and minimum of the data sets. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.0001$ (one-way ANOVA, Dunnett post-hoc test comparing drug concentration to no-drug control).

Figure 2.

$\alpha 6^*$ nAChRs mediate enhanced AMPAR function after *in vivo* ethanol administration. (A) Schematic of experimental procedure. Prior to ethanol injection, mice were habituated to handling by being handled once a day for three days, given a mock injection, and given a saline injection. Mice were given an ethanol or control saline injection 60 min prior to cutting brain slices containing the VTA. (B and C) Average (black trace) AMPA-evoked current traces from VTA DA neurons in (B) non-Tg and (C) $\alpha 6$ L9S brain slices cut 60 min after a mouse was given a control injection or an ethanol (0.5 g/kg or 2.0 g/kg) injection. Grey area fill indicates SEM for the average current trace. AMPA (100 μ M) was puff-applied while holding VTA DA neurons at -60 mV. Scale: 100 pA, 5 sec. (D) Summary of AMPA-evoked current responses from VTA DA neurons at a holding potential of -60 mV in brain slices from non-Tg and $\alpha 6$ L9S mice. The mean $\pm$ SEM is plotted while the dotted line indicates amplitude of baseline AMPA-evoked responses. *** $P < 0.001$ (one-way ANOVA, Dunnett post-hoc test comparing drug concentration to no-drug control); ## $P < 0.01$ (student's *t*-test comparing control from 0.5 g/kg in $\alpha 6$ L9S mice)

Figure 3.

AMPA function is enhanced by nicotine exposure in non-Tg and $\alpha 6L9S$ mice. (A) Schematic illustrating slice treatment procedure. Brain slices containing the VTA were prepared from non-Tg and $\alpha 6L9S$ mice and, after a 60 min recovery period, incubated in control holding solution or nicotine-containing holding solution for 60 min. Whole-cell patch clamp recordings were established in VTA DA neurons 60 min after slices were removed from nicotine. (B) Summary of AMPA-evoked current responses from VTA DA neurons at a holding potential of -60 mV in non-Tg and $\alpha 6L9S$ brain slices incubated in nicotine. The mean $\pm$ SEM is plotted while the dotted line represents average amplitude of baseline AMPA-evoked responses. * $P < 0.05$; *** $P < 0.001$ (one-way ANOVA, Dunnett post-hoc test comparing drug concentration to no-drug control)

Figure 4.

Nicotine and ethanol exposure enhance excitatory synaptic transmission in non-Tg and $\alpha 6L9S$ slices. (A) Schematic illustrating drug (nicotine or ethanol) exposure after cutting brain slices containing the VTA. After a 60 min recovery period, brain slices were incubated in a control holding solution or a solution containing nicotine or ethanol (concentrations indicated in (C) and (D) for 60 min. Slices were transferred out of ethanol for a 60 min washout period before initiating whole-cell recordings from VTA DA neurons. (B) Exemplary synaptic current evoked in a VTA DA neuron, showing AMPAR- and NMDAR-mediated components. AMPA EPSCs were measured at a command voltage of +40 mV (red trace) at the time corresponding to the peak of the AMPA EPSC elicited at a command voltage of -70 mV (blue trace). NMDA EPSCs were measured at +40 mV by averaging the membrane current during a 10 msec window 40 msec after the AMPA EPSC peak (the latter had decayed to baseline by this time, resulting in a current due completely to NMDARs). Scale: 40 pA, 10 msec. (C and D) AMPA/NMDA ratios in non-Tg (C) and $\alpha 6L9S$ (D) VTA DA neurons after exposure of slices to a control solution or to a solution containing the indicated drug. Data are shown as follows: grey boxes enclose the inner (25% to 75%) quartiles with the middle line indicating the median, and whiskers indicate the maximum and minimum of the data sets. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (one-way ANOVA, Dunnett post-hoc test)

Figure 5.

Subthreshold nicotine and subthreshold ethanol, when combined, enhance VTA AMPAR function in non-Tg slices. (A) Schematic of slice treatment procedure. 60 min after preparing brain slices containing the VTA from non-Tg mice, slices were incubated in holding solution containing both nicotine (100 nM) and ethanol (5 mM) for 60 min. AMPA-evoked currents were elicited and recorded after a 60 min wash-out period. In some experiments, slices were pre-incubated in either α CtxMII (100 nM) or varenicline (1 μ M) for 10 min before adding nicotine+ethanol for 60 min. Varenicline (1 μ M, 70 min) alone was also tested, as shown in (C). (B) Average (black trace) AMPA-evoked currents from non-Tg VTA DA neurons. Grey area fill indicates SEM for the average current trace. Slices were treated for 60 min with nicotine/ethanol in one of the following ways: 1) 5 mM EtOH only, 2) 100 nM nicotine only, 3) 5 mM EtOH plus 100 nM nicotine. AMPA (100 μ M) was puff-

applied while holding VTA DA neurons at -60 mV. Scale: 100 pA, 3 sec. (C) Summary of AMPA-evoked current amplitudes in VTA DA neurons in non-Tg brain slices. Data are shown as follows: grey boxes enclose the inner (25% to 75%) quartiles with the middle line indicating the median, and whiskers indicate the maximum and minimum of the data sets. $*P<0.05$; $**P<0.01$; (one-way ANOVA, Dunnett post-hoc test comparing the 5 mM EtOH group or the 100 nM nicotine group to the EtOH plus nicotine group).

Figure 6.

Subthreshold nicotine and subthreshold ethanol, when combined, enhance VTA AMPAR function in $\alpha 6L9S$ slices. (A) Schematic of slice treatment procedure. 60 min after preparing brain slices containing the VTA from $\alpha 6L9S$ mice, slices were incubated in holding solution containing both nicotine (3 nM) and ethanol (200 μM) for 60 min. AMPA-evoked currents were elicited and recorded after a 60 min wash-out period. In some experiments, slices were pre-incubated in either $\alpha CtxMII$ (100 nM) or varenicline (1 μM) for 10 min before adding nicotine+ethanol for 60 min. Varenicline (1 μM , 70 min) alone was also tested, as shown in (C). (B) Average (black trace) AMPA-evoked currents from $\alpha 6L9S$ VTA DA

neurons. Grey area fill indicates SEM for the average current trace. Slices were treated for 60 min with nicotine/ethanol in one of the following ways: 1) 200 μ M EtOH only, 2) 3 nM nicotine only, 3) 200 μ M EtOH plus 3 nM nicotine. AMPA (100 μ M) was puff-applied while holding VTA DA neurons at -60 mV. Scale: 100 pA, 3 sec. (C) Summary of AMPA-evoked current amplitudes in VTA DA neurons in α 6L9S brain slices. Data are shown as follows: grey boxes enclose the inner (25% to 75%) quartiles with the middle line indicating the median, and whiskers indicate the maximum and minimum of the data sets. $**P<0.01$; (one-way ANOVA, Dunnett post-hoc test comparing the 200 μ M EtOH group or the 3 nM nicotine group to the EtOH plus nicotine group).